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sensitive potassium channel proteins. Accordingly, the Examiner appears to believes the stated utilities are based entirely on conjecture from homologous polypeptides and that the asserted utilities are not specific to the claimed polypeptides, but rather based on family attributes. Furthermore, the Examiner alleges that neither the specification nor the art of record discloses any instances where disorders can be effected by interfering with the activity of the claimed polynucleotides thereby allegedly signifying that there is no "real world" context of use. In response, Applicants respectfully traverse.

The present invention is directed to nucleic acids that encode Slo3, a pH sensitive potassium channel expressed in spermatocytes. The claimed nucleic acids are full length cDNAs that are structurally and functionally characterized in the specification. As explained below, this rejection is improper because the teachings of the specification, taken in the context of what is known to those of skill in the art, make it clear that the claimed nucleic acids have utility. In the context of the present invention, one of skill would accept that the claimed nucleic acids actually encode a functional pH sensitive potassium channel expressed in spermatocytes (*see, e.g.*, Schrieber *et al., J. Biol. Chem. 273:3509-3516* (1998); Exhibit A). Furthermore, the claimed sequences demonstrate a real world utility, e.g., as targets for modulators of spermatocyte function, as shown in the Examples provided in the "Guidelines for Examination of Applications for Compliance with the Utility Requirement."

A. The reference SEQ ID NOS. all encode functional Slo3 potassium channels

In the present office action, the Examiner casts doubt on whether SEQ ID NOS:2, 17, and 19 all encode functional Slo3 potassium channels. In response, Applicants submit that SEQ ID NOS:2, 17, and 19 all encode functional Slo3 potassium channels. SEQ ID NO:2 encodes a functional mouse Slo3 potassium channel and SEQ ID NOs:17 and 19 encode functional human Slo3 potassium channels.

To demonstrate that one skilled in the art would believe that the claimed nucleic acids actually encode a pH sensitive potassium channel highly expressed in

spermatocytes, Applicants submit herewith a paper entitled Slo3, a Novel pH-sensitive K^+ channel from Mammalian Spermatocytes published in the February 6, 1998 issue of the peer reviewed journal, *The Journal of Biological Chemistry* (Schrieber *et al., J. Biol. Chem. 273:3509-3516* (1998); Exhibit A).

The paper, authored by two of the present inventors, describes the cloning and expression of the Slo3 homologue from mice. The mouse nucleotide sequence is provided as Figure 1 of the paper and is SEQ ID NO:2 of the present application. Structurally, the full length nucleotide sequence of mSlo3 (SEQ ID NO:2) encodes a protein of 1113 amino acids (SEQ ID NO:1) with a predicted molecular mass of 126 kDa. The authors also indicate that a human Slo3 homologue is present human testis.

The nucleotide sequence of the human Slo3 homologue is described in SEQ ID NOS:17 and 19 of the present application, and the amino acid sequence of human Slo3 is provided by SEQ ID NOS:16 and 18. As described in the specification on pages 62-63, the human Slo3 sequences were cloned from a human testis cDNA library using PCR primers based on the mouse sequence. The nucleotide sequence thus obtained was determined by the inventors of the present application to be highly homologous to the mouse Slo3 sequence, with high identity in the S1 region (part of the core domain). The specification describes that human Slo3 encodes a protein of similar size, sequence identity, and expression pattern as its mouse homologue (*see* Example 2 of the specification, in particular page 62, line 21-page 63, line 2). Northern analysis showed that human Slo3 was specifically expressed in the human testis (page 63, lines 1-2). The human Slo3 homologue is thus expressed in the human testis and shows high homology to the mouse Slo3 sequence. These results lead the inventors of the present application to designate the cloned human sequences as the human Slo3 homologues of mouse Slo3. As the human sequence is the counterpart or ortholog of the mouse Slo3 sequence, one of skill in the art would expect that the human sequence has the same functional attributes as the mouse sequence.

The acceptance and subsequent publication of the paper in the *Journal of Biological Chemistry*, as well as the data presented in the specification, demonstrates that

those of skill in the art believe that the claimed nucleic acids are Slo3 nucleic acids that encode a Slo3 pH sensitive potassium channel. The claimed sequences, therefore, represent functional Slo3 potassium channels.

B. Claims directed to fully characterized proteins or nucleic acids that encode them satisfy the utility requirements of 35 U.S.C. § 101.

As evidenced by the submitted reference, the Slo3 family of pH sensitive potassium channels is a recognized family of pH potassium channels.

The claimed Slo3 nucleic acids are fully characterized both structurally and functionally. The Slo3 nucleic acid sequences are claimed by reference to shared structural features, i.e., percent identity to the sequences disclosed in SEQ ID NO:2, SEQ ID NO:17 and SEQ ID NO:19. The Slo3 nucleic acids are also claimed by reference to shared functional features, *i.e.*, the ability to form a pH sensitive potassium channel highly expressed in spermatocytes activated by changes in intracellular pH and membrane potential.

As described in the present specification, full length cDNAs that encode a Slo3 potassium channel were cloned, and mRNA expression patterns were determined for each clone. Functional assays were performed to determine the biological activity of the clones. When expressed in *Xenopus* oocytes according to standard methodology, the encoded channel produces currents that are sensitive to both pH and voltage. The Slo3 potassium channel has a unit conductance of approximately 80-120 pS when in functional tetrameric form, is capable of transporting potassium ions, and has increased potassium ion transporting activity above an intracellular pH of 7.1. Furthermore, routine methods for electrophysiological characterization of potassium channels are described in the specification.

According to the recently promulgated "Guidelines for Examination of Applications for Compliance with the Utility Requirement," a characterized protein has utility. This standard is made evident from Example 8 of the guidelines. In Example 8, a compound A is disclosed to inhibit enzyme XYZ, a well known enzyme, *in vitro*. The

specification states that the compound A can be used to treat diseases caused or exacerbated by enzyme XYZ. No such diseases are named. The first claim is directed to compound A. The second claim is directed to a method of treating a disease caused or exacerbated by enzyme XYZ consisting of administering an effective amount of compound A to a patient. In the subsequent analysis, claim 2 is deemed to not be supported by a real world context of use. This result is because neither the specification nor the art of record discloses any disease or conditions caused or exacerbated by enzyme XYZ and therefore, the asserted utility is seen as a method of treating an unspecified, undisclosed disease or condition, which does not define a "real world" context of use. Claim 1, however, is accepted as having utility because claim 1 is directed to a compound that inhibits an enzyme and enzymes have well established utility in the art, i.e., catalyzing certain reactions.

This example can be compared to the present application. The present application claims compound A, e.g., nucleic acids that encode a potassium channel, as compared to a compound that inhibits an enzyme. The specification states that compound A, the potassium channel, can be used to treat diseases affected by changes in pH and membrane potential in the body, as compared to Compound A, enzyme inhibitor, which can be used to treat disease affected by the enzyme. In Example 8, claims directed to compound A were found to be useful even though there was no disclosure of specified disease that could be treated. Accordingly, even if the Examiner believes, contrary to Applicants disclosure and the enclosed declaration, that Slo3 is not involved in sperm capacitation, a claim directed to the compound, e.g., the nucleic acid, has utility. The nucleic acids have utility because they encode potassium channels and potassium channels, like enzymes, have a well established utility in the art. Applicants therefore respectfully request that the rejection be withdrawn.

Rejection under 35 U.S.C. § 112, first paragraph, enablement (in light of the utility rejection)

Claims 1, 4, 5, 8, 9, 26-27, and 45-47 remain rejected under 35 U.S.C. § 112 as not being enabled since the invention is allegedly not supported by either a clear asserted utility or a well established utility. The Applicant respectfully traverses the rejection. As described above, the invention claimed in the present application is supported by a specific, substantial, and credible utility. The specification provides methods of identifying the claimed nucleic acids, methods of activating and blocking Slo3 channels, and methods of identifying agonists and antagonists of Slo3 channels. Accordingly, the Applicant requests that the rejection under 35 U.S.C. § 112 be withdrawn.

Rejection under 35 U.S.C. § 112, first paragraph, enablement

Claims 1, 4, 5, 8, 9, 26-27, and 45-47 are rejected under 35 U.S.C. § 112 as allegedly lacking enablement for reciting moderate stringency conditions. Applicants respectfully traverse. However, to expedite prosecution, Applicants have amended the claims to recite stringent hybridization conditions.

As identified in the Patent Office and the Federal Circuit, whether undue experimentation is required by one skilled in the art to practice to invention is determined by considering factors such as the amount of guidance presented in the application, the state of the prior art, and the presence of working examples. *Ex parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Int. 1985); *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). As described in Wands, a "considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which experimentation should precede." *Wands*, 8 USPQ2d at 1404 (quoting *In re Jackson*, 217 USPQ 804 (Bd. Pat. App. & Int. 1982).

The present invention describes a family of Slo3 nucleic acids which (1) functionally encode monomers that form potassium channels having a unit conductance of approximately 80-120 pS and having increased potassium channel current activity

above approximately intracellular pH of 7.1 when expressed in a *Xenopus* oocyte; and (2) structurally hybridize under stringent conditions to reference full-length Slo3 nucleic acids.

Hybridization and amplification methods for the identification of nucleic acids are well known to those of skill in molecular biology and are described in the specification (*See* pages 23-24) These structural elements therefore provide adequate guidance for routine identification of the nucleic acids of the invention.

Applicants direct the Examiner's attention to the sections of the specification which disclose functional assays for identification of nucleic acids encoding Slo3 potassium channel polypeptide monomers of the invention. On page 11, line 33 to page 12, line 10, the specification teaches expressing a Slo3 polypeptide encoded by a putative nucleic acid of the present invention in *Xenopus* oocytes and examining the polypeptide's functional characteristics. A nucleic acid with similar nucleotide identity to Slo3 that encodes a polypeptide that forms a potassium channel with Slo3 characteristics, e.g., a unit conductance of approximately 80-120 pS and increased potassium channel current activity above approximately intracellular pH of 7.1, is a Slo3 nucleic acid of the present invention. Conversely, a sequence that diverges even by only one nucleic acid, is not a species of Slo3 if it doesn't form a potassium channel with Slo3 characteristics when expressed. This expression assay allows one of skill in the art to routinely identify the claimed nucleic acids without undue experimentation. Therefore, the functional characteristics of the proteins encoded by the claimed nucleic acids would allow one of skill in the art to identify operable embodiments and exclude inoperable embodiments.

Another method of detecting and quantifying Slo3 is disclosed in the specification on pages 37-42. This section teaches using standard immunological assays known to those of skill in the art to detect and quantify Slo3. Again, persons of skill in the art, after reading the specification, would know to express sequences similar to Slo3, without undue experimentation, in order to determine if the sequences do, in fact, encode Slo3 potassium channels.

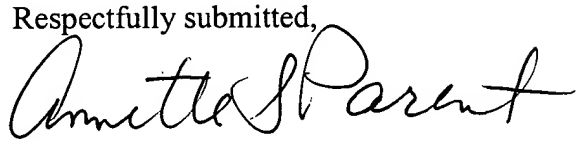
Finally, as previously described in the response mailed October 10, 2000, Applicants clearly meet the PTO guidelines for enablement, which set forth the standard for the scope of enablement when a large number of possible embodiments exists. Thus, undue experimentation is not required to practice the claimed invention. Applicants therefore respectfully request that the rejection be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at .

Respectfully submitted,



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APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

1. (four times amended) An isolated nucleic acid encoding a polypeptide monomer of a pH sensitive potassium channel, the monomer:

(i) forming a potassium channel having a unit conductance of approximately 80-120 pS and having increased potassium channel current activity above approximately intracellular pH of 7.1, when the monomer is expressed in a *Xenopus* oocyte; and

(ii) encoded by a nucleic acid that selectively hybridizes under [moderate stringency hybridization conditions to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2, 16, or 18, wherein the hybridization reaction is incubated at 37°C in a solution comprising 40% formamide, 1 M NaCl, and 1% SDS and washed at 45°C in a solution comprising 1x SSC] highly stringent hybridization conditions to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2, , SEQ ID NO:17, or SEQ ID NO:19, wherein the hybridization reaction is incubated at 42°C in a solution comprising 50% formamide, 5x SSC, and 1% SDS and washed at 65°C in a solution comprising 0.2x SSC and 0.1% SDS.

Exhibit A

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Slo3, a Novel pH-sensitive K^+ Channel from Mammalian Spermatocytes*

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Potassium channels have evolved to play specialized roles in both excitable and inexcitable tissues. Here we describe the cloning and expression of Slo3, a novel potassium channel abundantly expressed in mammalian spermatocytes. Slo3 represents a new and unique type of potassium channel regulated by both intracellular pH and membrane voltage. Reverse transcription-polymerase chain reaction, Northern analysis, and *in situ* hybridization show that Slo3 is primarily expressed in testis in both mouse and human. Because of its sensitivity to both pH and voltage, Slo3 could be involved in sperm capacitation and/or the acrosome reaction, essential steps in fertilization where changes in both intracellular pH and membrane potential are known to occur. The protein sequence of mSlo3 (the mouse Slo3 homologue) is similar to Slo1, the large conductance, calcium- and voltage-gated potassium channel. These results suggest that Slo channels comprise a multigene family, defined by a combination of sensitivity to voltage and a variety of intracellular factors. Northern analysis from human testis indicates that a Slo3 homologue is present in humans and conserved with regard to sequence, transcript size, and tissue distribution. Because of its high testis-specific expression, pharmacological agents that target human Slo3 channels may be useful in both the study of fertilization as well as in the control or enhancement of fertility.

Many ion channels respond to membrane depolarization or intracellular ligands. In the Slo1 channel, originally cloned from *Drosophila*, these features are combined in a channel that opens in response to both depolarization and intracellular calcium increases (1, 2). Slo1 channels cloned from mouse and human show strong conservation of sequence and functional properties (3–7). One proposed role of the Slo1 channel is to provide negative feedback for the entry of calcium into cells via hyperpolarization-induced closure of voltage-dependent calcium channels. Perhaps because of the versatility of this mechanism, Slo1 channels are expressed in many tissues where voltage-gated calcium channels are present, including brain, skeletal and smooth muscle, auditory hair cells, pancreas, and adrenal gland (8–15). In contrast to other multigene voltage-gated K^+ channel families, Slo1 has remained the sole functionally characterized representative of its family (16). Here we describe mSlo3,[1] a pH- and voltage-dependent Slo family member prominently expressed in mouse spermatocytes. Despite similarity in sequence of mSlo3 to mSlo1, mSlo3 is insensitive to calcium over a wide concentration range. Thus, the unifying characteristics of the Slo gene family of channels may be a dual sensitivity to membrane voltage and a variety of intracellular factors, such as $[H^+]$ for mSlo3.

The uniquely abundant expression of mSlo3 in developing spermatocytes presents further interesting questions. Spermatocytes require proteins tailored to fulfill roles unique to the process of germ cell development and fertilization. Cellular signaling in spermatic cells is tightly regulated to prevent inappropriate activation of the irreversible steps that prepare the sperm to fertilize the oocyte. Many of these steps are triggered and coordinated by changes in membrane potential and intracellular Ca^{2+} concentration and pH. Because of the central importance of these events in development, many efforts have been made to identify the specific proteins, including ion channels, which regulate spermatic function. In particular, there have been reports of channels present in spermatocytes (19, 20), including voltage-dependent calcium channels (21–24). In addition, a cyclic nucleotide-gated channel has been directly cloned from testis (25). **The mSlo3 channel exhibits a unique combination of voltage dependence and sensitivity to pH that may be important for spermatic function.**

MATERIALS AND METHODS

Cloning—By tBlastn (NCBI), an EST (GenBank™ accession number AA072586) was identified by homology to the C-terminal "tail" of mSlo1 (17). The EST originated from a mouse promyelocytic WEHI-3 cell line cDNA library. A ^{32}P-labeled 1,254-bp PCR product generated from the EST pBluescript plasmid (Genome Systems) was employed to isolate cDNA clones from a WEHI-3 library (Stratagene) by hybridization. The oligonucleotides used to generate the probe were 5′-GTGGATGATACCGACATGCTGGAC-3′ (sense) and 5′-GAGACCACCTCTCTCCCGTGTCGT-3′ (antisense). mSlo3 expression in the WEHI-3 cell line is apparently anomalous; all isolated cDNAs were inappropriately spliced or truncated, and PCR analysis of the WEHI-3 cDNA bank using combinations of primers complementary to mSlo3 and vector sequence indicated that complete cDNAs were not represented. Subsequent screening of a mouse testis cDNA library (Dr. Graeme Mardon) yielded cDNAs that extended to the putative initiator methionine, as shown in Fig. 1. The reading frame was closed upstream from the initiator methionine. A full-length cDNA was constructed from two overlapping cDNAs. The presence of full-length transcripts in testis corresponding to this cDNA was verified by RT-PCR from total testis RNA. The entire

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF039213.

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[1] The abbreviations used are: mSlo3, mouse Slo3; RT-PCR, reverse transcription-polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; TEA, tetraethylammonium; bp, base pairs(s); kb, kilobase(s).

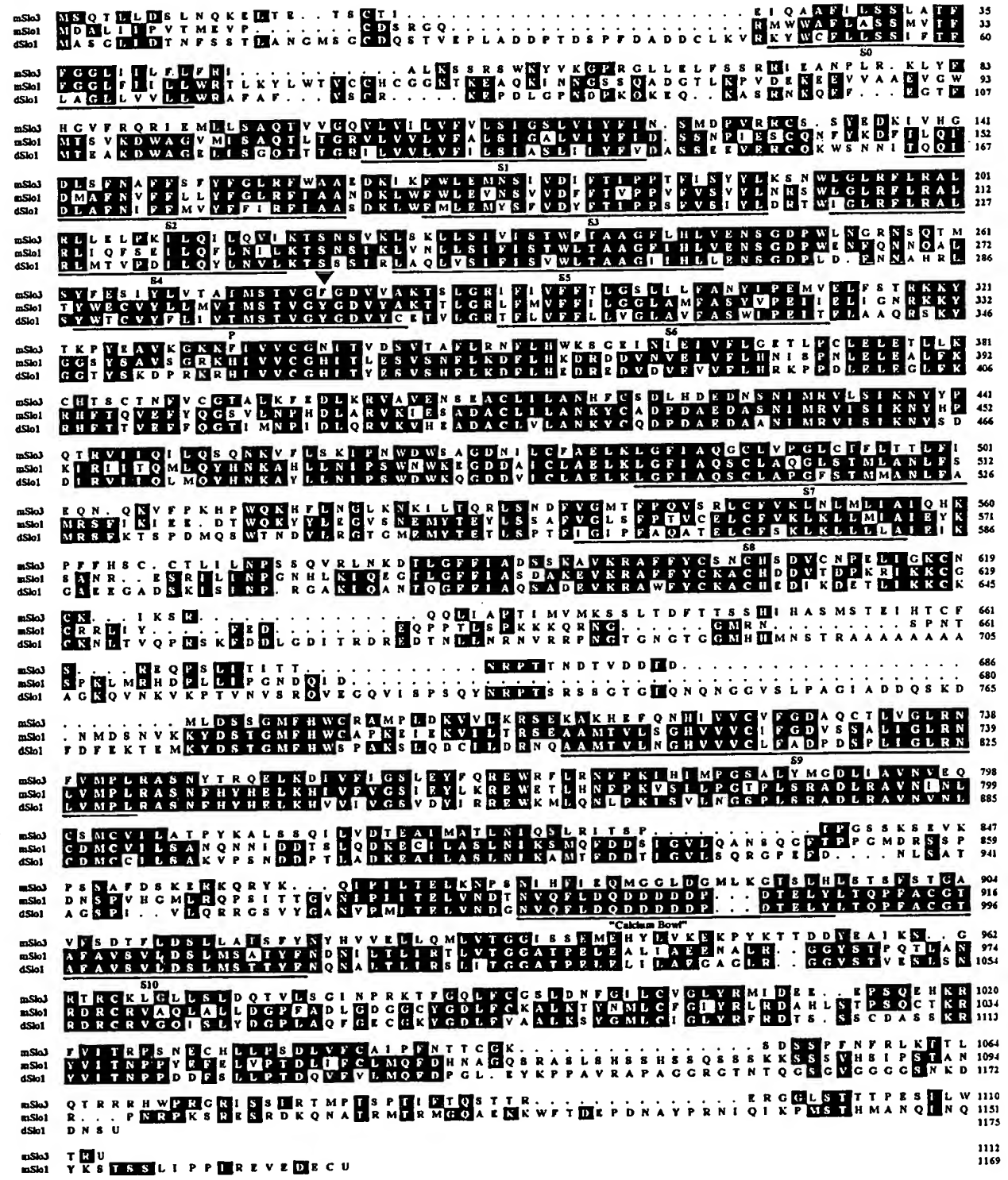

FIG. 1. **Primary sequence of mSlo3.** *A*, alignment of the primary sequence of mSlo3 with BK Ca^{2+}-activated K^+ channels mSlo1 (mouse) and dSlo1 (*Drosophila*). Hydrophobic segments are designated S0 through S10 (3, 27). An *arrowhead* indicates a phenylalanine residue (F) in the pore region critical for ion selectivity (see text). The region designated "Calcium Bowl" has been implicated in the regulation of mSlo1 by calcium (18). The overall core and tail organization of Slo1 has been conserved in Slo3 (17). mSlo1 (mbr5) (3) and dSlo1 (splice variant A2C2E2G5I0) (1, 2) sequences are shown. Slo2, not shown, is a more distantly related sequence (16). *B*, Kyte-Doolittle (49) hydrophilicity plots of mSlo3 and mSlo1.

cDNA was sequenced in both directions. For expression in *Xenopus* oocytes, a Kozak initiator sequence (26) was introduced by PCR, and the entire open reading frame was subcloned into the pOocyte-Xpress vector (17). cRNA was generated using the mMessage mMachine kit (Ambion); details of cRNA synthesis were as previously published (17).

RT-PCR—For each tissue, Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) was used on 5.0 μg of total RNA primed with 25 μM random hexanucleotides (Boehringer Mannheim) and 200 μM dNTPs at 42 °C for 1 h. 0.1% of each first strand synthesis was assayed by PCR using 1.0 μM oligonucleotide primers, 200 μM dNTPs, and 0.0075 units of KlenTaq, cycling 30 times. Reaction products were electrophoresed on 1.5 and 3.0% agarose gels using standard

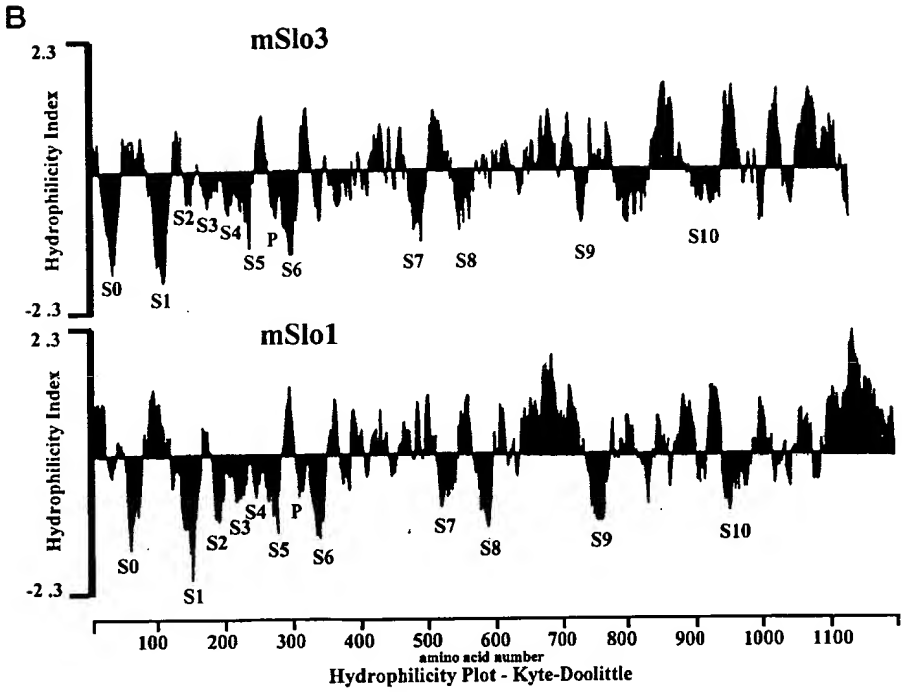

FIG. 1—*continued*

Tris-borate (TBE) buffer and visualized by staining with ethidium bromide. PCR primer pairs used were 1) mSlo3 (S4 to S5), 5′-CTCGAACTCCCTAAAATCTTACAGAT-3′ (sense) and 5′-TTCCGTTGAGCCAGGGGTCACCAGAATT-3′ (antisense) to generate a 156-bp product; 2) mSlo3 (S8 to S9), 5′-TCTGCTTTGTGAAGCTAAATCT-3′ (sense) and 5′-TTTCAAAGCCTCTTTAGCGGTAA-3′ (antisense) to generate a 690-bp product; 3) mSlo3 (S9 to S10), 5′-TTATGCCTGGATCTGCACTCTACATG-3′ (sense) and 5′-ATAGTTTCCGTCTACTACCGAAA-3′ (antisense) to generate a 221-bp product; 4) human β-actin, 5′-GATGATATCGCCGCGCTCGTCGTCGAC-3′ (sense) and 5′-TCGGTCCAGGTCTGCGTCCTACCGTAC-3′ (antisense) to generate a 535-bp product.

Northern Blot Analysis—Total RNA was isolated from freshly dissected mouse tissue using Trizol (Life Technologies, Inc.). 20 μg of total RNA from each tissue was electrophoresed on a 1% agarose denaturing gel using MOPS-formaldehyde buffer then transferred to nitrocellulose. The human tissue blot was obtained commercially (CLONTECH). A PCR product generated using primers 5′-CGGAAACGTCATGTACAATCGAAATCCA-3′ (sense) and 5′-TTCCGTTGAGCCAGGGGTCACCAGAATT-3′ (antisense) was labeled using random hexanucleotides (Boehringer Mannheim). Both human and mouse blots were hybridized and washed under standard high stringency conditions and exposed to x-ray film for 4–16 h. After hybridization with mSlo3 probes, blots were rehybridized with a human β-actin probe to verify RNA loading.

In Situ Hybridization—Testes from white mice >30 days old were dissected, frozen, sectioned immediately with a cryostat, collected on slides, and stored at −20 °C. A partial mSlo3 cDNA (approximately 1 kb, corresponding to coding sequence for residues 170–510) was subcloned into pBluescript II KS⁺ (Stratagene). T3 and T7 RNA polymerase (Stratagene) were used to synthesize [^{33}P]UTP-labeled antisense and sense probes, respectively, from linearized plasmid. Slides were hybridized overnight at 55 °C. After washing, slides were dipped in NTB-2 liquid emulsion (Eastman Kodak Co.), air-dried, and placed in light-protected boxes at 4 °C for 10 days.

Electrophysiology—cRNA (40 nl at approximately 1 μg/μl) was injected into mature *Xenopus* oocytes; recordings were made 1–8 days later. For whole cell recording, medium nd96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) supplemented with 1 mM DIDS (to block endogenous chloride currents) was employed as a bath solution or modified as noted in the figure legends. Patches were perfused with either zero Ca^{2+}-EGTA solutions (160 mM potassium gluconate, 34 mM KOH, 10 mM HEPES, and 10 mM EGTA) or Ca^{2+}-containing (184 mM potassium gluconate, 10 mM KOH, 10 mM HEPES, 200 μM hemicalcium gluconate) solutions. HCl was used to adjust the pH. The pipette solution contained 0.5 mM potassium gluconate, 0.5 mM KCl, 1.1 mM KOH, 10 mM HEPES, 159 mM sodium gluconate, and 2 mM hemimagnesium gluconate, pH 7.1. Whole cell recordings were obtained using the two-electrode voltage clamp TEV-200 amplifier (Dagan). Patch currents were recorded on either an Axopatch 1B or 200A amplifier (Axon) and digitized at either 3.4, 5, or 10 kHz. Data acquisition and analysis programs were CCURRENT and CQUANT (Dr. Keith Baker) or pClamp6 (Axon). Single-channel conductance was determined from the slope of the unitary current amplitude *versus* voltage relation. The tail current amplitude *versus* voltage data (see Fig. 4) were fit with the sum of two independent Goldman-Hodgkin-Katz current equations. Each equation has the form $I = PV_mF^2/RT \times ([S]_i - [S]_o \times \mathrm{EXP}(-V_mF/RT))/1 - e^{-V_mF/RT}$, where S is either K^+ or Na^+: P, the permeability of Na^+ or K^+, was allowed to vary freely. $[S]_i$ and $[S]_o$ represent ion concentrations inside and outside the oocyte, respectively; V_m is the membrane potential; F, R, and T have their usual meanings. Reversal potential *versus* varying extracellular cation concentration (see Fig. 4) was fit with the Goldman-Hodgkin-Katz equation: $E_{reversal} = RT/F \times \ln([K^+]_o + P \times [Na^+]_o)/([K^+]_i + P \times [Na^+]_i)$, where P is the Na^+ to K^+ permeability ratio. Fits were performed using Sigmaplot (Jandel). Recordings were made at room temperature except tail currents, which were recorded at 11 °C using a Peltier device (Cambion).

RESULTS

Cloning and Primary Sequence of mSlo3—We isolated the mSlo3 cDNA from a testis cDNA library based on its homology to the large conductance calcium-activated (BK) potassium channel, mSlo1 (see "Materials and Methods"). The probe was generated from an expressed sequence tag identified in the GenBank™ data base. The new channel was termed mSlo3 (the "m" prefix denoting mouse derivation, "h" referring to the human homologue, hSlo3). Fig. 1 illustrates that the 1,112 amino acid mSlo3 protein is similar along its entire length to the 1,169 amino acid mSlo1 protein (3) as well as *Drosophila* Slo1 (*dSlo1*) (1, 2). The hydrophilicity profiles of both sequences indicate 11 hydrophobic segments, S0 through S10 (Refs. 3 and 27 and Fig. 1*B*). As with mSlo1, these can be divided into core and tail domains. Similarity between mSlo3 and mSlo1 is greatest in the core domain. The mSlo3 core (S0 through S8: mSlo3 residues 35 through 641) shares 56 and 50% identity with mSlo1 and dSlo1 cores, respectively. (Interspecies homologues mSlo1 and dSlo1 share 62% identity.) The mSlo3 tail (S9 and S10: mSlo residues 686–1136) shares 39% identity with

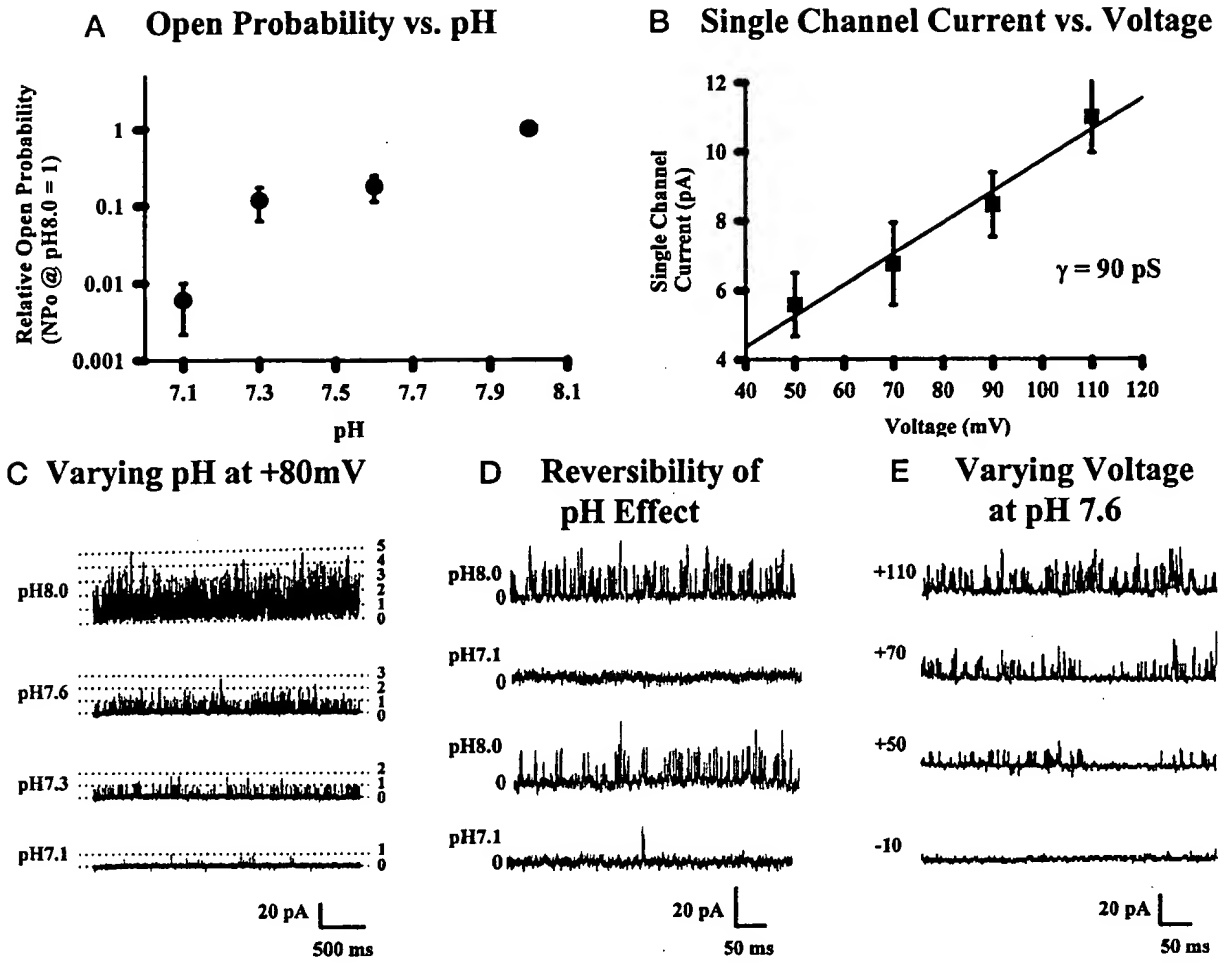

FIG. 2. **mSlo3 channel sensitivity to voltage and pH shown in inside-out patches.** *A*, open probability increases at higher pH at a constant voltage (+80 mV). NP_o was calculated assuming the presence of five channels in the patch. The data was normalized to the NP_o at pH 8. The cytoplasmic surface was exposed to recording solution at indicated pH (n = 3). *B*, single-channel conductance calculated from the slope of single channel current amplitude plotted against membrane voltage in asymmetric K^+ (cytoplasmic side: 194 mM K^+, 0 mM Na^+; extracellular side: 2.1 mM K^+, 159 mM Na^+; n = 4). The conductance in symmetric 213 mM K^+ was calculated to be 106 picosiemens (data not shown). *C*, currents from a single patch showing higher activity at higher pH; the open times of single channel events are brief. *D*, reversibility of pH effect. A single patch is shown alternately exposed to pH 8.0 and pH 7.1. The sequence is from top to bottom. *E*, channel activity increases at positive potentials at a constant pH (pH 7.6).

mSlo1 and dSlo1, respectively. (The interspecies homologues mSlo1 and dSlo1 share 68% identity in this region.) A linker region having no significant conservation is found between S8 and S9. More detailed comparison of mSlo3 and mSlo1 sequences implied two functional properties of mSlo3. 1) The absence of the "calcium bowl" (18) suggested that mSlo3 may be activated by factors other than Ca^{2+}. 2) In the K^+-selective pore, a GFG motif, rather than the typical GYG, suggested differences in ionic selectivity between the two channels (28, 29).

Heterologous Expression of mSlo3 cRNA Produces Voltage- and pH-sensitive Currents—When expressed in the *Xenopus* oocyte expression system, mSlo3 cRNA produced currents that were sensitive to both pH and voltage. This was demonstrated in observations of single channel behavior (Fig. 2), macroscopic currents in the patch configuration (Fig. 3), and whole cell oocyte currents recorded in the two-electrode voltage clamp mode (Fig. 4). In patches where unitary openings could be resolved, mSlo3 single-channel currents were observed to have very brief open times. Currents were sensitive to intracellular pH. Currents were small or absent at a pH_i of 7.1 or lower, whereas raising pH_i resulted in sharp increases in channel activity (Fig. 2). As shown in Fig. 2, the effect of changing pH was completely and repeatedly reversible. Macroscopic currents in the inside-out patch configuration behaved consistently with single channel behavior, despite channel rundown, which occurred over the course of minutes (Fig. 3). Patches exposed to pH 7.1 produced virtually no current. However, raising the pH to 8.0 resulted in macroscopic currents that responded to depolarization and exhibited little or no inactivation. In the whole cell configuration, mSlo3 currents resembled a voltage-dependent delayed rectifier and, consistent with macroscopic currents in the patch clamp configuration, showed little or no inactivation (Fig. 4). This behavior indicates that the internal pH of oocytes must be higher than pH 7.1, consistent with previous reports that reveal resting *Xenopus* oocyte pH near 7.5 (30, 31). Based on this assumption, it was predicted that the manipulation of internal pH would either reduce the amplitude of observed whole cell currents (after acidification) or increase the amplitude of currents (after alkalization). Thus, acidification of the oocyte using bicarbonate-based bath solution (30) reduced the amplitude of currents (Fig. 4*A*, *middle*), whereas alkalization by ammonium chloride (31) increased the amplitude of mSlo3 currents even after attenuation by bicarbonate (Fig. 4*A*, *right*).

The effect of calcium ion on the gating of mSlo3 currents was

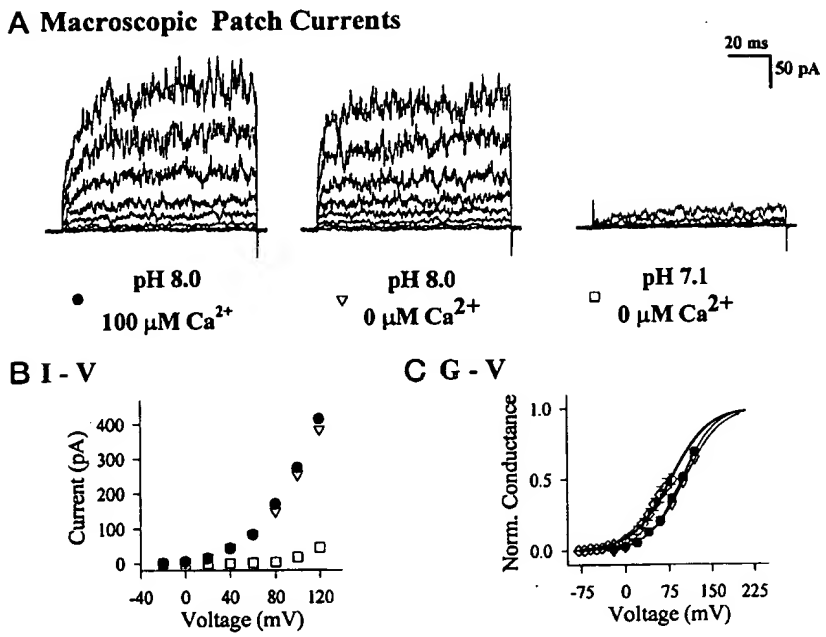

FIG. 3. **Macroscopic current traces showing sensitivity to pH but insensitivity to calcium ion.** *A*, current traces from the same patch exposed to pH 8 with Ca^{2+} (*filled circles*), pH 8 without Ca^{2+} (*triangles*), or pH 7.1 without Ca^{2+} (*squares*). *B*, corresponding current-voltage (*I-V*) relations for the traces in A. *C*, calculated G-V curves for the patch in *A* exposed to 100 μM Ca^{2+} (*filled circles*) or 0 Ca^{2+} (*open triangles*), both at pH 8.0. For comparison, the averaged whole-cell conductance-voltage relation was plotted (*open diamonds*; $n = 12$). This relation falls approximately 25 mV to the left of the macroscopic patch curve. Each set of points was fitted with a single Boltzmann equation. Because none of the data points approach roll-over (saturation of the conductance), the Boltzmann fits must be regarded as an estimate only. The current shown in 0 μM Ca^{2+} at pH 7.1 (*A*) was restored in amplitude when the patch was again exposed to pH 8. The slight decrease in current amplitude between the two sets of traces at pH 8 was most likely due to current rundown. Holding potential was −40; on-line leak subtraction was employed.

investigated by observing macroscopic currents in the inside-out patch configuration and exposing the cytoplasmic surface to various concentrations of Ca^{2+}. It was found that macroscopic patch currents were unaffected by changing [Ca^{2+}] from nominally zero (10 mM EGTA solution with no added calcium) to 100 μM Ca^{2+} perfused on the intracellular face (Fig. 3). As a control, the same solutions were used on inside-out patches containing mSlo1 channels instead of mSlo3. Using an identical voltage protocol, large macroscopic currents were evoked at 100 μM, but currents were absent in zero calcium conditions (data not shown; see also Ref. 18).

mSlo3 single-channel conductance was observed to be 90 picosiemens as measured in asymmetric potassium concentrations (Fig. 2) and 106 in symmetric high K^+ (data not shown). This is considerably lower than that seen for mSlo1 (270 picosiemens) measured in symmetric high k^+ (3). Unlike mSlo1, mSlo3 is relatively insensitive to tetraethylammonium. mSlo3 channels were tested for external TEA sensitivity in the whole cell, two-electrode recording mode. Oocytes expressing mSlo3 cRNA were stepped from −40 to +80 mV while several concentrations of TEA were added sequentially to the bath. A plot of current *versus* TEA concentration at +60 mV revealed an EC_{50} of 49 mM (data not shown). In contrast, mSlo1 is approximately 350-fold more sensitive (having an EC_{50} of 0.14 mM) (3).

Slo3 Currents Show Relaxed K^+ Selectivity—One conspicuous difference in sequence between mSlo3 and mSlo1 occurs in a region implicated in ion selectivity. All channels with high selectivity for potassium over sodium have a GYG sequence motif in the "P" region (28, 29). In contrast, mSlo3 has a GFG at this location (Fig. 1, residue 279). It was previously shown in a Shaker K^+ channel that substitution of F (Phe) for Y (Tyr) in the GYG sequence decreased selectivity for K^+ over Na^+ (32). This suggested that mSlo3 could have less selectivity for K^+ over Na^+ than most potassium channels. To test this possibility, we analyzed the reversal potential for mSlo3 current tails at various concentrations of external potassium and sodium ion (Fig. 4). Our results showed that mSlo3 is less selective for K^+ over Na^+ than Slo1, having a P_K/P_{Na} of approximately 5 *versus* >50 for Slo1 (3, 5).

Slo3 Expression Is Prominent Only in Spermatocytes—Figs. 5, *A* and *B*, show results from RT-PCR performed on RNA from brain, skeletal muscle, lung, liver, kidney, and heart; only testis produced a positive signal. Northern blots using total RNA from the same tissues gave a robust signal after only 4 h of exposure. A transcript of approximately 4 kb was seen only in testis (Fig. 5). A longer exposure (18 h) failed to reveal bands from any additional tissues (data not shown).

A Human Slo3 Homologue Is Present in Testis—Northern analysis of a human tissue blot also revealed a band at 4 kb, indicating the presence of a human Slo3 homologue of a conserved transcript size. The human Northern blot similarly indicated a restricted tissue distribution (Fig. 5). The small message size is unusual for a gene encoding such a large protein. Apparently, there may be as little as 700 bp of 5′- and 3′-untranslated regions. This small message size contrasts with the larger message size of mSlo1, which is more than twice as large in brain (3). Preliminary sequence data indicates hSlo3 is highly conserved at the molecular level.

In Situ Hybridization—In testis, *in situ* hybridization revealed that mSlo3 message is expressed in the seminiferous tubules with the signal directly over developing spermatocytes (Fig. 6). The message is most abundant in the inner segments of these rings, corresponding to positions over maturing spermatocytes and possibly to early spermatids. The outermost regions of these rings contain spermatogonia, the stem cell from which spermatocytes are derived. The absence of hybridization (*arrow* in Fig. 6) in the peripheral areas suggests that expression is restricted to later stages of spermatogenesis. The absence of message in the interstices between rings also suggests that the nerves, blood vessels, and connective tissue in these regions of the testis probably lack expression of mSlo3.

DISCUSSION

Sensitivity to both intracellular alkalization and membrane depolarization distinguishes mSlo3 from other cloned channels. Previous reports demonstrate that other channels from various tissues are sensitive to pH_i, but their functional properties as well as their tissue distributions differ from mSlo3. One example is the acid-sensing channel (ASIC), which opens upon intracellular acidification (33). These channels are present in dorsal root ganglia and are involved in pain sensation. The ASIC channel is in the amiloride-sensitive Na^+ channel degenerin family and is not similar to K^+ channels. In contrast, TASK and RACTK1 are in the K^+ channel superfamily but lack

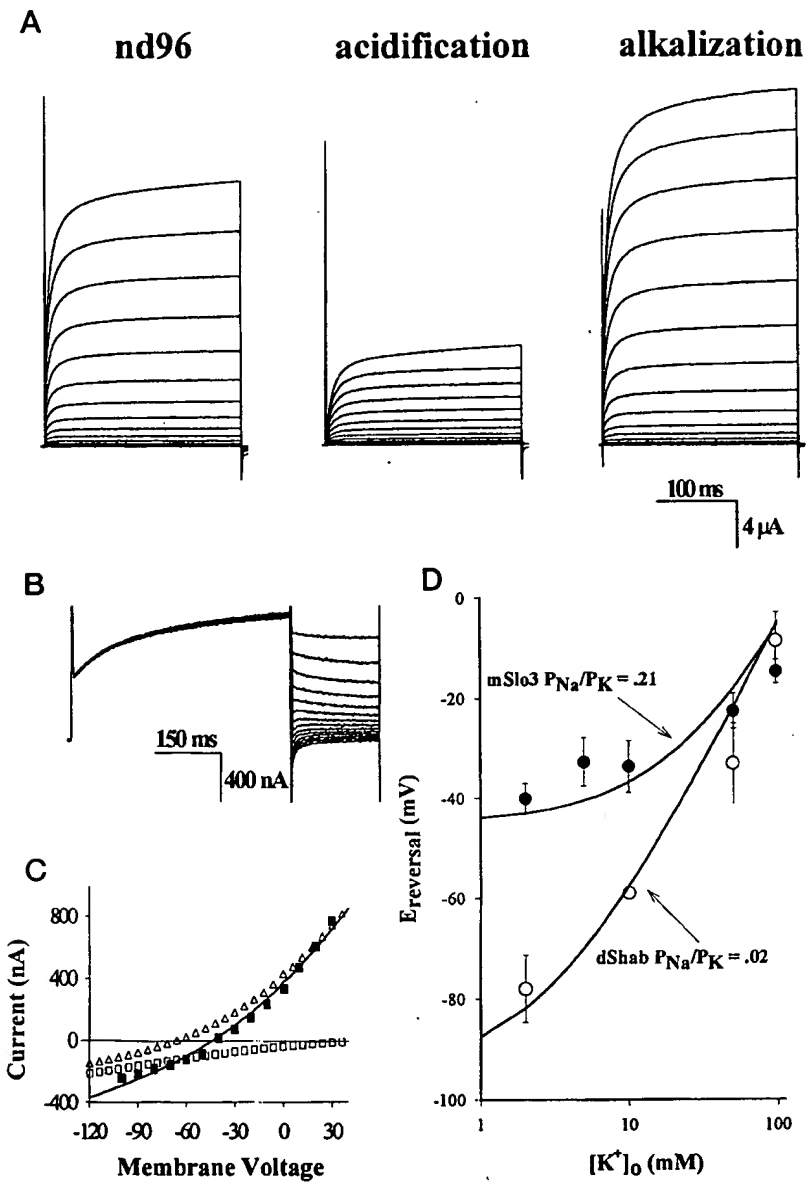

FIG. 4. **mSlo3 whole cell currents from *Xenopus* oocytes.** *A*, manipulation of intracellular pH alters current amplitude. *Left*, control currents at start of experiment. *Middle*, diminished current amplitude after intracellular acidification (12.5-min perfusion with $NaHCO_3$ replacing NaCl in nd96). *Right*, recovery during alkalization (10-min perfusion with nd96 supplemented with 30 mM NH_4Cl). Voltage families are from −80 to +60 mV in 10-mV increments. *B*, representative tail currents used to determine channel selectivity. *C*, tail current amplitude *versus* voltage plotted for the currents shown in *B*. Instantaneous currents at the time of the voltage jump were calculated from exponential fits of tail currents extrapolated back to time zero. *Filled squares* indicate currents plotted *versus* test potential. A Goldman-Hodgkin-Katz current equation was fitted (*solid line*) to the tail current-voltage relation. The fit showed a Na^+/K^+ permeability ratio of 0.15. The calculated underlying K^+ (*triangles*) and Na^+ (*open squares*) currents are also shown. (For the example shown in *B* and *C*, bath ion concentrations were 10 mM $[K^+]$, 88 mM $[Na^+]$, 11 °C.) *D*, reversal potentials plotted at different potassium concentrations to illustrate the relatively low selectivity of mSlo3 for K^+ over Na^+. mSlo3 data is shown compared with the more highly selective dShab channel (50). Reversal potentials were determined by measuring tail currents in varying external $[K^+]$; $[Na^+]$ was also varied, so that the total monovalent concentration ($[K^+]$ + $[Na^+]$) was 98 mM. Points were fitted with a Goldman-Hodgkin-Katz equation where the Na^+/K^+ permeability ratio, *P*, was allowed to vary freely. For mSlo3 reversal potentials at 2, 5, 10, 50, and 98 $[K^+]$, n = 6,3,5,6,7, respectively; for dShab at 2, 10, 50, and 98, n = 3, 1, 3, 3.

the S4 voltage sensor and do not respond to membrane potential changes. TASK is a recently cloned, four-transmembrane domain channel responsive to extracellular proton concentrations in the physiological range; it is presumed that the TASK channel contributes to the resting K^+ conductance (34). The RACTK1 kidney K^+ channel, a two transmembrane domain channel, is activated by intracellular alkalization but is voltage-insensitive (35). In contrast, mSlo3 as well as other Slo family channels may have evolved to respond to both voltage as well as other intracellular factors; calcium in the case of mSlo1 and pH in the case of mSlo3.

The properties of mSlo3 make it difficult to work with. In addition to its very short mean open time and low open probability (P_o) at physiological voltages, there is also a nagging rundown problem to contend with. It is especially difficult to get patches with sufficient channels for macroscopic current analysis. Because very large depolarizations are required to elicit activity of the channel, g/g_{max} plots do not approach saturation and the Boltzmann fitted to these plots can only serve as an estimate of channel behavior. However, from these approximate Boltzmanns, the whole-cell current V_{50} appears to be +72 ± 4.9 mV (mean ± S.E.; n = 12; Fig. 3*C*). The slope of these *V versus* g/g_{max} relations is extremely shallow (approximately 16 mV/e-fold). Thus, the base of the curve is in a physiological range even though g_{max} is not attained until +200 mV. Because of this shallow slope, it appears that at least some channels must be open in the physiologically relevant voltage range. Perhaps the mSlo3 channel is designed to provide small increments of current. Alternatively, our heterologous expression system might be missing something that affects gating properties, perhaps a cytoplasmic factor or auxiliary subunit native to sperm that contributes to the gating of this channel. One hint that cytoplasmic factors may be important is the fact that the estimates of V_{50} from whole cell voltage clamp recordings are shifted approximately −25 mV relative to the macroscopic patch current (Fig. 3*C*). This may suggest that another factor does indeed influence channel gating. A missing factor could permit the mSlo3 channel to function more prominently within the pH and voltage range present in spermatocytes or mature sperm.

The mechanism of proton sensitivity in mSlo3 is unknown. In ROMK inward rectifier channels, a titratable lysine residue has been shown to confer reversible proton block when pH is reduced below 7.5, both in whole cell recordings from oocytes

FIG. 5. **Expression of mSlo3 transcripts is largely restricted to the testis.** *A*, RT-PCR of mouse brain, heart, skeletal muscle, kidney, testis, lung, and liver with mSlo3-specific primers, with (+) and without (−) the addition of reverse transcriptase. An expected 156-bp product is detected only in testis RNA. Similar results were obtained with two additional mSlo3 primer pairs specific to S8–S9 and S9–S10 regions (data not shown). *B*, control RT-PCR assays with β-actin-specific primers produce an expected 537-bp product from all tissues. Additional negative controls with genomic DNA (10 ng) or primers alone are also shown. *C*, Northern blot analysis of total RNA (20 μg) from mouse brain, heart, skeletal (*sk*) muscle, kidney, testis, lung, and liver reveals an abundant mSlo3 transcript only in testis, with an approximate size of 4 kb. *D*, Northern blot analysis of polyadenylated RNA (2 μg) from human tissues (spleen, thymus, prostate, testis, uterus, small (*sm*) intestine, colon, and leukocytes) with mSlo3 reveals a hybridizing mRNA species only in testis, with an approximate size of 4 kb. Controls are shown below; the same blots were hybridized with a human β-actin probe.

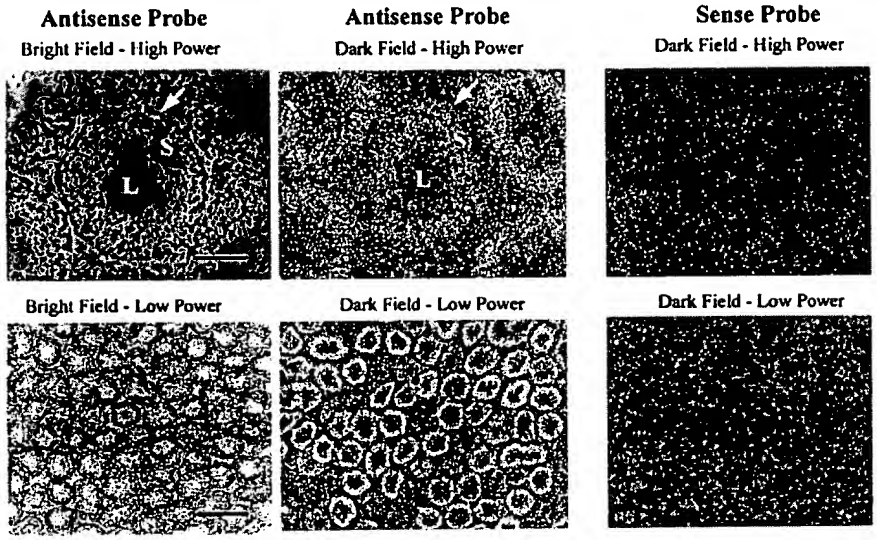

FIG. 6. ***In situ* hybridization localizes expression of mSlo3 mRNA in seminiferous tubules.** *Upper row*, high magnification light micrographs of adult mouse testis. *Left*, annular cross-section of a seminiferous tubule (*arrow*), containing mostly developing spermatocytes (*S*). Spermatic germ cells and primary spermatocytes are at the outer edges, whereas more mature spermatocytes are found near the lumen (*L*). Less abundant Sertoli and Leydig cells are difficult to distinguish. *Middle*, the same field shows intense hybridization signal (*white puncta*) in more mature spermatocytes. The interstices between tubules are unlabeled. *Right*, control section hybridized with an mSlo3 sense probe. *Lower row*, low magnification micrographs. *Left*, many seminiferous tubule cross-sections are visible. *Middle*, dark field of a similar section shows labeling of annular masses of developing spermatocytes. Scattered tubules with low hybridization signal may reflect segments at earlier stages of spermatogenesis. *Right*, a similar control section hybridized with sense probe. *Scale bar*: *top*, 100 μm; *bottom*, 500 μm.

exposed to bicarbonate-containing media or in perfused detached patches (30, 36). It is possible that mSlo3 is inhibited by protons by a similar mechanism. In Slo1 channels, raising $[Ca^{2+}]_i$ increases channel activity by enabling the channel to activate at more hyperpolarized voltages (8, 9). Preliminary data from mSlo3 suggests that changing the proton concentration may not alter the voltage range of activation of the channels. Although Slo1 Ca^{2+} sensitivity is altered at different pH_i, this effect may depend on the alteration of calcium affinity by protonating residues involved in calcium binding (37). It is not clear if or how this may relate to mSlo3 gating.

mSlo3 represents a new member of the Slo family of channels. Surprisingly, although these channels share a great deal of sequence identity, the functional features of these channels, exceptionally large conductance, calcium sensitivity, and high selectivity for K^+ over Na^+, do not appear to be highly conserved Slo family characteristics. The structure of the Slo1 channel resembles a voltage-dependent channel core with an appended tail region that plays a modulatory role in gating by sensing Ca^{2+} (17, 18). We previously demonstrated the existence of two calcium sensing sites in the mSlo1 channel, the calcium bowl, a high affinity site in the tail region, and a lower affinity site at a location that has not yet been determined (18). The lack of Ca^{2+} sensitivity in mSlo3 implies that neither of

these two sites are present. Properties associated with the core domain also differ between Slo3 and Slo1. Slo3 is approximately 350-fold less sensitive to external TEA. Underlying this difference may be the absence of a tyrosine residue at a site reported to be critical to external TEA sensitivity (38). Rather than a tyrosine residue as in TEA-sensitive channels, the mSlo3 sequence has a valine at the corresponding position (valine at position 283). The highly selective toxins that block BK channels, charybdotoxin (39) and iberiotoxin (40), did not affect mSlo3 currents at 50 and 20 nM, respectively (data not shown). In contrast, an S4 voltage-sensing domain is present in both Slo1 and Slo3, corresponding to voltage sensitivity, which the channels share (41). The pairing of voltage sensitivity with sensitivity to a variety of intracellular factors may be the unifying feature of the Slo family.

Several observations indicate uniquely abundant expression in testis: a high density of labeling in *in situ* experiments, a strong signal in Northern analysis using total RNA, and high representation in a testis cDNA library ("Materials and Methods"). These data cannot exclude very low levels of expression or spatially restricted patterns of expression within the other tissues examined. However, if mSlo3 expression is indeed largely restricted to spermatocytes, it may be that the pairing of sensitivity to pH and voltage is designed to fulfill a unique role in spermatocytes. All proteins utilized by the mature sperm are synthesized during spermatogenesis, as mature sperm lack translational activity. Thus, although the mSlo3 protein has not yet been identified in mature sperm, robust transcription in developing spermatocytes makes it likely that the channel is present at these later stages. The unlikely alternative is that mSlo3 is utilized only during a narrow window of time in sperm development. Assuming its presence in mature sperm, the unusually high permeability ratio of sodium to potassium could allow multiple roles for mSlo3 channels that depend on the extracellular environment that sperm encounter. In high external Na^+ or K^+, Slo3 channels could have a depolarizing influence; conversely, where these ions were low in the external environment, the open channel would be hyperpolarizing. It is intriguing to speculate on the role of mSlo3, since both alkalization and depolarization are components of the signaling pathway during both sperm capacitation and the acrosome reaction, two essential steps preceding sperm fertilization of the oocyte (21, 42–45). Between mating and fertilization, sperm undergo capacitation, a process that later enables them to fertilize the oocyte. Capacitation involves an increase in cytosolic pH (pH_i), which promotes metabolic and swimming activity (42, 46–47). An increase in pH_i, changes in membrane potential, and a rise in cytoplasmic $[Ca^{2+}]$ trigger the acrosome reaction upon contact with the oocyte (21, 43, 48). As the sperm membrane depolarizes, voltage-gated calcium channels open, permitting the entry of calcium and thereby triggering the release of the acrosomal granule (23). Although the role of Slo3 in these processes remains speculative at this time, it is plausible that this channel plays a role in coordinating these events by directly linking cellular pH and membrane voltage.

Future investigation may focus on substances known to affect sperm function, which may use this channel as a target. Finally, agents that block or open this channel may be useful in the study or control of fertility.

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